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Bactericidal Effect of Doxycycline Associated with Lysosomotropic Agents on *Coxiella burnetii* in P388D$_1$ Cells

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There is no consistently reliable treatment for endocarditis resulting from chronic *Coxiella burnetii* infection, the causative agent of Q fever. Although certain antibiotics are recommended on the basis of their in vitro bactericidal activities, results of therapy with these antibiotics are often disappointing. To evaluate whether the currently recommended antibiotic susceptibility tests for *C. burnetii* give misleading results because of continued division of uninfected cells, thereby resulting in the dilution of infected cells and, hence, a false picture of antibiotic efficacy, we blocked cell division during antibiotic susceptibility testing with cycloheximide. Using this new method, we found that the currently recommended antibiotics for the treatment of Q fever, doxycycline, pefloxacin, and rifampin, did not reduce the ratio of infected to noninfected cells (either L929 or P388D$_1$) by 9 days postinfection. To test the hypothesis that this lack of antibacterial activity is due to antibiotic inactivation by the low pH of the phagolysosomes in which *C. burnetii* is found, we used alkalinizing lysosomotropic agents (chloroquine or amantadine) concurrently with doxycycline. This resulted in the sterilization of *C. burnetii* infection in P388D$_1$ cells. This finding seems to confirm our suspicion that the acidic conditions of the phagolysosomes in which *C. burnetii* is located inhibit antibiotic activity. This inhibition can be reversed in vitro when lysosomotropic alkalinizing agents are used.

Infections of humans and other animals by *Coxiella burnetii*, the causative agent of Q fever, have been reported worldwide. Following the acute form of the disease, the most serious complication is endocarditis, which has a mortality rate of 37% (9). Early antibiotic susceptibility studies (6, 8, 12) with embryonated egg assays suggested that tetracycline and doxycycline should be used for treatment. Because these antibiotics were demonstrated to have only a bacteriostatic effect, a 3-year treatment duration was recommended (9). By using this treatment regimen, relapses of infection have frequently been observed. Also, *C. burnetii* has been successfully isolated from cardiac valves 1 year after a full course of treatment with tetracycline (13). Later, a model to determine the antibiotic susceptibility of *C. burnetii* in patients with chronic infections was described by Yeaman and colleagues (10, 11, 15, 16) by using persistently infected L929 cells. By this method, doxycycline was found to be ineffective, but several quinolones (pefloxacin, ofloxacin, ciprofloxacin) and rifampin were interpreted to have a bactericidal effect. As a result of those studies, quinolones and rifampin in association with doxycycline have commonly been used in the treatment of endocarditis as a result of Q fever. Unfortunately, clinical data have not supported the reported in vitro bactericidal effects of these drugs. We have noted a relapse in a patient after 1 year of therapy with doxycycline and pefloxacin for Q fever endocarditis and, in addition, have isolated *C. burnetii* from the cardiac valve of a patient after 4 months of doxycycline and ofloxacin therapy (P. Y. Levy, M. Drancourt, J. Etienne, J. C. Auvergnat, J. Beytout, J. M. Sainty, F. Goldstein, and D. Raoult, submitted for publication).

In order to explain these discrepancies, we developed a new method for the determination of antibiotic susceptibility in patients with chronic Q fever infections. Since we suspected that continued cell division in the previously described antibiotic susceptibility test led to a dilution of the number of infected cells and, hence, a false impression of antibiotic efficacy, we used cycloheximide to inhibit cell division during our susceptibility testing, as is done in *Chlamydia* assays (14). Using this new method, we failed to demonstrate a bactericidal effect with the tetracyclines, the quinolones, or rifampin. To explain this lack of efficacy and in an attempt to determine an effective antibiotic regimen for the treatment of Q fever endocarditis, we decided to investigate the possibility that it is the acidity (pH 5) of the phagolysosomes, where *C. burnetii* is located (2, 4), which inhibits antibiotic activity. Such a possibility has been suggested in the past for *Staphylococcus aureus* (7). In this report, we describe the concurrent use of lysosomotropic alkalinizing agents and antibiotics in a new in vitro antibiotic susceptibility test.

MATERIALS AND METHODS

In this study the Nine Mile strain of *C. burnetii* was used. Infected cells, either P388D$_1$ or L929, as described previously (1, 15), were grown in 25-cm^2 tissue culture flasks (Eagle minimal essential medium, 10% fetal bovine serum, 2 mM glutamine) at 37°C in a 5% carbon dioxide incubator. Cells that were gently scraped from the bottom of a flask were found to be 90% infected by Gimenez staining on three successive passages and were used for antibiotic susceptibility testing. Pefloxacin (Roger Bellon, Paris, France), erythromycin (Abbott, Paris, France), doxycycline (Pfizer, Paris, France), rifampin (Lepetit, Paris, France), chloroquine (Specia, Paris, France), and amantadine (Du Pont, Paris, France) were prepared in aqueous solutions of 1 mg/ml and then divided into portions and frozen at −20°C until use. On day 0 of the antibiotic challenge experiments, pefloxacin, erythromycin, and doxycycline were added to the culture media to give final concentrations of 1, 2, or 4 μg/ml, while chloroquine and amantadine were used at 1 μg/ml. To inhibit cell division during antibiotic susceptibility

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testing, cycloheximide was prepared as described above and was used at a final concentration of 1 μg/ml.

Comparison of in vitro antibiotic tests in dividing and nondividing cells. For comparisons of controls and each test antibiotic, 90% infected L929 and P388D$_1$ cells were used. Three infected flasks containing media with no drugs were used as controls, while three flasks were exposed to cycloheximide only. The susceptibility of *C. burnetii* to pefloxacin (4 μg/ml), doxycycline (4 μg/ml), rifampin (4 μg/ml), erythromycin (4 μg/ml), chloroquine (1 μg/ml), and amantadine (1 μg/ml) was then evaluated in cells which were prevented from dividing by the concurrent addition of cycloheximide to the growth media. Each susceptibility test was repeated three times. From day 3 post-drug inoculation, cell infection rates were evaluated each day until day 10 postinfection by Gimenez staining of cells that were scraped from each flask. The cell medium was changed on days 3 and 6 post-drug inoculation.

Assessment of antibiotic susceptibilities on nondividing cells in the presence of lysosomotropic alkalinizing agents. Microtiter plates (96-well) were used for the assessment of antibiotic susceptibilities on nondividing cells in the presence of lysosomotropic alkalinizing agents. A 100-μl suspension of 10^5 P388D$_1$ *C. burnetii*-infected cells per ml was added to each well. A total of 50 μl of tissue culture was added to each well on the first line of the plate, and these acted as untreated controls. To the remaining wells, 50 μl of cell culture medium was added together with cycloheximide to give a final concentration of 1 μg/ml. No drugs were added to line 2 of the plate but antibiotics were added to the next nine lines of the plate. In lines 3, 4, and 5, pefloxacin was used at 1, 2, and 4 μg/ml, respectively. In lines 6 to 8, erythromycin was used, and in lines 9 to 11, doxycycline was used at the concentrations given above for pefloxacin. Finally, the first 6 and last 6 wells of each line received chloroquine and amantadine, respectively, to a final concentration of 1 μg/ml. Two wells of each line were sampled and stained with Gimenez on days 3, 6, and 9 post-drug inoculation.

Confirmation of microtiter plate results for doxycycline. Results of our in vitro antibiotic susceptibility tests on microtiter plates with doxycycline were confirmed by using 25-cm^2 flasks of 90% *C. burnetii*-infected P388D$_1$ cells blocked with cycloheximide. No additional drugs were added to three such flasks, and they acted as controls. Doxycycline was added at a concentration of 4 μg/ml to the media in three other flasks. The lysosomotropic alkalinizing agents chloroquine and amantadine were each added to three flasks together with doxycycline (4 μg/ml). The tissue culture media were changed every 3 days post-drug inoculation, and at that time cells were scraped from the flasks for microscopy by using Gimenez staining. One flask contained doxycycline alone (4 μg/ml), one flask contained doxycycline and chloroquine (1 μg/ml), and one flask contained doxycycline and amantadine (1 μg/ml).

RESULTS

In all cases, cells that were infected with *C. burnetii* and treated with cycloheximide died within 3 days, but control cells remained alive with 90% infection rates and could be passaged every 3 days. Infected cells treated with cycloheximide and doxycycline, pefloxacin, rifampin, or chloroquine survived for 9 days but did not multiply and were as infected as control cells to which no drugs were added to the growth medium. Cells cultured with erythromycin or amantadine only died within 3 days, as was the case with control cells treated with cycloheximide only.

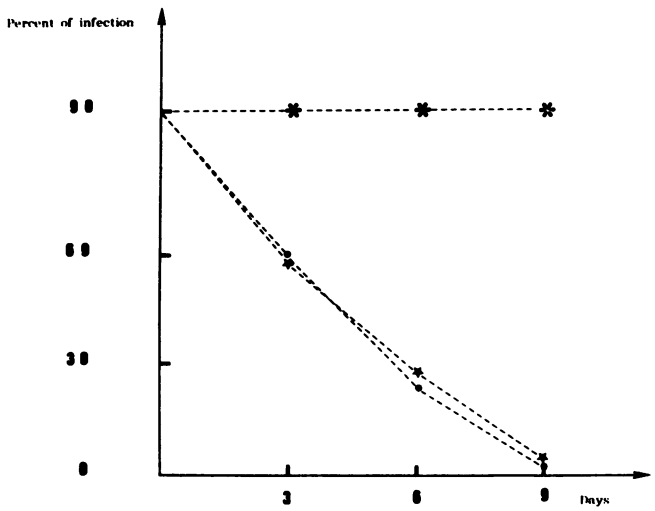

FIG. 1. Effect of doxycycline alone (4 μg/ml) (*) and in combination with chloroquine (1 μg/ml) (★) and amantadine (1 μg/ml) (●) in P388D$_1$ 90% *C. burnetii*-infected cells blocked with cycloheximide.

In the experiments that were performed to assess the combined effect of antibiotics and alkalinizing lysosomotropic agents in microdilution cell culture plates, the only efficient combination in reducing the ratio of infected to uninfected cells was doxycycline (2 or 4 μg/ml) combined with either chloroquine or amantadine.

The results presented above were confirmed in 25-cm^2 flasks. On day 3, cells treated with doxycycline (4 μg/ml) alone were 90% infected, cells treated with doxycycline and chloroquine were 60% infected, cells treated with doxycycline and amantadine were 55% infected, and all control cells treated with cycloheximide only died. On day 6, the results were 90, 25, and 30%, respectively, and on day 9, the results were 90, 0, and 0%, respectively (Fig. 1). When cells were incubated with doxycycline and subcultured with either chloroquine or amantadine, no sign of *C. burnetii* infection could be detected.

DISCUSSION

The antibiotic susceptibility findings presented here are not in accord with in vitro data obtained either in embryonated eggs (6, 8, 12) or in persistently infected L929 cells (10, 11, 15, 16). Our results with cycloheximide-treated L929 cells were dramatically different from the results with untreated L929 cells presented previously (10, 11, 15, 16). Cycloheximide-treated *C. burnetii* cells without antibiotics died in 3 days. Untreated infected cells maintained a 90% *C. burnetii* infection rate and could be passaged every 3 days. The addition of doxycycline, pefloxacin, or rifampin to the cycloheximide-treated cells enabled the cells to survive but did not reduce the ratio of infected to noninfected cells. From these results, we conclude that these antibiotics are bacteriostatic and not bactericidal. The fact that cycloheximide and antibiotic-treated cells survived, while cycloheximide-treated cells died, indicates that the cause of cell death in the later category of cells was due to *C. burnetii* replication and cell lysis. The same results were obtained with P388D$_1$ cells. These are mouse macrophages in which persistent *C. burnetii* infections have been described (1). These results led us to speculate that the lack of a bactericidal

effect was due to the acidity within the phagolysosome. Some basis exists for this idea, as Hackstadt and Williams (4) have reported a bacteriostatic effect of chloroquine against *C. burnetii*, supposedly because of alkalinization of the phagolysosome. Chloroquine and amantadine, the drugs used in our study, are lysosomotropic agents; that is, they are concentrated by living cells into lysosomes. Because of their alkalinizing properties, they raise the pH of the normally acidic lysosomes (3). We used chloroquine and amantadine at concentrations similar to those found in the sera of human patients treated with these drugs for various conditions. In our in vitro antibiotic testing method for *C. burnetii*, we confirmed the findings of Hackstadt and Williams (4) that chloroquine is bacteriostatic.

In our experiments to establish whether alkalinization of the phagolysosome in which *C. burnetii* is found could result in a better antibiotic efficacy, we tested the effects of the association of doxycycline, pefloxacin, and erythromycin with the lysosomotropic agents chloroquine and amantadine. Pefloxacin and erythromycin continued to show only a bacteriostatic effect. However, in association with both chloroquine and amantadine, doxycycline was bactericidal (Fig. 1).

The data presented in this report indicate that we have found an in vitro model of infection to explain why quinolones and doxycycline may not be efficient in sterilizing some patients with chronic Q fever endocarditis. Our results suggest that the reason why doxycycline has a bacteriostatic and not a bactericidal effect could be related to the very low pH of the phagolysosome. The modification of the acidity of the lysosomes of patients with chronic Q fever may increase antibiotic efficacy, thereby shortening the courses of treatment and improving successful treatment rates. The reason why only doxycycline becomes bactericidal with lysosomotropic alkalinizing agents could be related to the fact that the pH at which it has maximal activity is low (pH 6.8). Perhaps the modification of the lysosomal pH may have been insufficient in our experiments to improve the efficacy of pefloxacin and erythromycin. This hypothesis is being investigated in our laboratory.

The generalization of the concept that modification of the pH of lysosomes allows for more effective antibiotic treatment of other facultative intraphagolysosomal bacteria remains to be demonstrated but should be of interest.

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